JOURNAL OF PROTEOMICS 75 (2012) 2697–2706

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Proteomic analysis provides new insight into the chicken eggshell cuticle

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ARTICLE INFO

Article history:
Received 6 February 2012
Accepted 15 March 2012
Available online 27 March 2012

Keywords:
Antimicrobial
Cuticle
Eggshell
Ovocalyxin
SERPIN

ABSTRACT

The cuticle is the outermost layer of the avian eggshell, whose protein constituents remain virtually unknown. We hypothesize that cuticle components play a major role in microbial resistance, since eggs with incomplete or absent cuticle are more susceptible to bacterial contamination. In this study we extracted proteins from the outermost non-calcified layer of the cuticle of chicken eggs and subjected them to LC/MS/MS proteomic analysis. We identified 47 cuticle proteins with high confidence and reproducibility. Two proteins, similar to Kunitz-like protease inhibitor and ovocalyxin-32 (a carboxypeptidase A inhibitor), were the most abundant of the cuticle proteins. A number of proteins known to have antimicrobial activity in the egg were detected (lysozyme C, ovotransferrin, ovocalyxin-32, cystatin, ovoinhibitor) as well as possible new candidates (myeloperoxidase, ovocalyxin-36 and members of the SERPIN family). This is the first comprehensive report of cuticle proteome, a starting point to determine cuticle function and the molecular basis of its antimicrobial properties.

1. Introduction

Integrated defense strategies that operate at biomineralized barriers are a hallmark of sophisticated biological structures. One example, although not well understood, is the calcified eggshell that is essential for reproduction in birds and reptiles. The avian calcified shell is a complex structure that is deposited while the forming egg is retained in the distal oviduct (uterus / shell gland) during an extended period. Genetics controls the shell permeability for metabolic gases and water, which depends on the characteristics of its pores - number, density, branching pattern and caliber. Ultimately, a cuticle is deposited onto the eggshell surface during the final phase of egg formation [1]. The cuticle mainly consists of proteins (>85%) and possesses two layers: the innermost is mineralized and contains hydroxyapatite-containing vesicles deposited during the final phase of eggshell calcification (termination); the outermost remains non-mineralized [2,3]. The cuticle is distributed unevenly over the surface of the egg; its thickness ranges from 5 to 10 μm [4]. The cuticle covers the calcified shell and fills the entry to its pores (up to 50 μm in depth), creating a barrier which inhibits water movement across the shell and prevents dehydration of the egg interior [5]. Moreover, the cuticle physically excludes bacterial penetration of pores and limits microbial colonization on the egg's surface [6,7]. Eggs with absent or incomplete cuticle are more susceptible to bacterial contamination [8]. Cuticle desiccation during egg storage or incubation leads to cracks that expose eggshell pores and leave the egg vulnerable to contamination by pathogens [9]. A pathogen-free egg is extremely important for avian reproduc-

Abbreviations: BCA, bicinchoninic acid; BPI, bactericidal/permeability-increasing; BPTI, bovine pancreatic trypsin inhibitor; emPAI, exponentially modified protein abundance index; GO, gene ontology; OC, ovocleidin; OCX, ovocalyxin; pBLAST, protein basic local alignment search tool; SERPIN, serine proteinase inhibitor; DAVID, database for annotation, visualization and integrated discovery; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein.

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doi:10.1016/j.jprot.2012.03.019

tion and survival of the developing embryo, in addition to food safety of the nutritious unfertilized egg intended for human consumption. Eggs and egg-containing foods are the main vehicles for *Salmonella enteritidis* intoxication (poisoning) [10], in addition to potential contamination by *E. coli*, *Pseudomonas* sp., *Micrococcus* sp. and various other bacterial strains [7]. In order to understand the molecular basis for the antimicrobial function of the eggshell cuticle and gain further overall insight into cuticle function, we performed a comprehensive proteomic analysis of the outermost (non-calcified) layer of the cuticle.

2. Materials and methods

2.1. Cuticle protein extraction

Unwashed freshly laid eggs from Lohmann LSL-Lite chickens were obtained from Laviolette Poultry Farm, St-Isidore, Ontario. In two independent trials, intact eggs (n=5) were individually extracted in sterile plastic bags containing 2 mL of extraction solution: 1% SDS or 1% SDS, 2 mM DTT. The egg surface was manually massaged for 5 minutes with extraction solution to solubilize the cuticle layer. An empty plastic bag was treated with extraction solution to assess possible contamination arising from the extraction method (none was detected). The individual extraction samples were transferred to Amicon Ultra-4 Centrifugal Filter Devices (molecular weight cutoff of 3000 Da, Millipore Corporation, Billerica, MA) and centrifuged at 4000 rpm to concentrate the dilute cuticle extracts. The protein concentration of each concentrated extract was measured with the bicinchoninic acid (BCA) assay (Thermo Scientific, Hampton, NH) before pooling for electrophoresis. Pooled extracts were resolved by SDS-PAGE using a precast 4-12% BisTris gel (Invitrogen, Carlsbad, CA). The gels were sectioned and sent to the Proteomics Platform of the Eastern Quebec Genomics Centre (Laval, QC) for LC/MS/MS analysis (services include in-gel digestion, mass spectrometry and Mascot database searching, Sections 2.2–2.5).

2.2. Protein in-gel digestion

Gel sections were placed in 96-well microplates, washed with water and digested with trypsin on a MassPrep liquid handling robot (Waters, Milford, MA) [11,12]. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 126nM of modified, sequencing grade, porcine trypsin (Promega, Madison, WI) at 58 °C for 1 h. The digestion products were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile, pooled, vacuum centrifuged dried and then resuspended into 7 μL of 0.1% formic acid. Only 2 μL of the resuspended extracts were analyzed by mass spectrometry.

2.3. Mass spectrometry

Online reversed-phase nanoscale capillary liquid chromatography and electrospray mass spectrometry (ES MS/MS) were used to separate and analyze peptide samples. The experiments were performed with a Thermo Surveyor MS pump connected to a LTQ linear ion source (ThermoFisher, San Jose, CA). Peptide separation occurred on a Self-Pack PicoFrit column (New Objective, Woburn, MA) containing Jupiter packing material (Phenomenex, Torrence, CA) 5u, 300A, C18, 10 cm×0.075 mm internal diameter. Peptides were eluted by a 2–50% solvent B (acetonitrile, 0.1% formic acid) linear gradient in 30 minutes, at 200 nL/min (obtained by flow-splitting) while the mass spectra were obtained using Xcalibur software version 2.0 and a data dependent acquisition mode. Collision-induced dissociation of the seven most intense ions followed each full scan mass spectrum (400 to 2000 m/z). Other parameters include an enabled dynamic exclusion (30 second exclusion duration) function and a relative collisional fragmentation energy set to 35%.

2.4. Database searching

The MS/MS results were analyzed with Mascot (Matrix Science, London, UK; version 2.2.0), searching the uniref-100.2010.06.Gallus.gallus.9031database (37461 entries), with trypsin digestion. Search parameters included a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 Da. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification, while oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed. The same procedure was repeated with Mascot set up to search uniref-100_2010_06_Bacteria_2 database (5093156 entries) to assess possible bacterial contamination of the unwashed egg surface by bacteria from the farm environment or chicken feces. No peptides of bacterial origin were detected.

2.5. Criteria for protein identification

Validation of MS/MS based peptide and protein identifications were performed using Scaffold (version Scaffold-3_00_08, Proteome Software Inc., Portland, OR) and were accepted if they were identified at $p \leq 0.05$ probability, specified by the Protein Prophet algorithm [13,14]. Protein identifications also required at least 2 unique peptides to be accepted. The principles of parsimony were utilized to group proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone.

2.6. Bioinformatic analysis

The relative quantification of the identified proteins were calculated using the exponentially modified protein abundance index (emPAI)=10^($N_{observed}/N_{observable}$)−1, where $N_{observed}$ is the amount of unique parent ions obtained and $N_{observable}$ corresponds to the amount of peptides expected after digestion with trypsin [15]. The protein Basic Local Alignment Search Tool (pBLAST) was used to align proteins identified by Scaffold against the non-redundant protein sequence database for the species *Gallus gallus* (9031). Potential signal peptides in the predicted full-length protein were assessed using the SignalP 4.0 Server (www.cbs.dtu.dk/services/SignalP) and were only accepted as valid if both the neural network and hidden Markov models identified a signal peptide ($p \leq 0.05$). Gene Ontology (GO) term enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/